Bioorganic & Medicinal Chemistry Letters 22 (2012) 6862–6866

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Isolation, characterization and cytotoxic activity of benzophenone glucopyranosides from Mahkota Dewa (*Phaleria macrocarpa* (Scheff.) Boerl)

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ARTICLE INFO

Article history:
Received 10 June 2012
Revised 31 August 2012
Accepted 12 September 2012
Available online 20 September 2012

Keywords:
Mahkota Dewa
Benzophenone glucopyranoside
Synthesis
Bioactivity

ABSTRACT

Two benzophenone glucopyranosides have been isolated from the nut shell part of Mahkota Dewa. The structures were identified as 2,4′,6-trihydroxy-4-methoxy-benzophenone-2-*O*-β-D-glucoside (Mahkoside A) and 2,4′,6-trihydroxy-4-methoxy-6″-acetyl-benzophenone-2-*O*-β-D-glucoside (Mahkoside B). Mahkoside B was recognized as a novel compound. Furthermore, a series of benzophenone glucopyranoside derivatives (compounds **3**–**18**) were synthesized and their bioactivities were characterized. Our results demonstrated that compound **18** has significant cytotoxicity against two esophageal cancer cell lines, stomach cancer cell line and prostate cancer cell line, with IC_{50} less than 10 μM, indicating its potential activity against cancer cells.

Mahkota Dewa [*Phaleria macrocarpa* (Scheff.) Boerl] is mainly distributed in Southeast Asia region, especially in Indonesia. Due to its antihistamine, anti-oxidation and antitumor effects, it has been used to treat many diseases, including cancer, impotency, haemorrhoids and diabetes mellitus, et al.[1] Mahkota Dewa is also used as a major ingredient in some healthy drink or tea in Indonesia. Mahkoside A (2,4′,6-trihydroxy-4-methoxy-benzophenone-2-*O*-β-D-glucoside) was previously identified by our laboratory as a component in Mahkota Dewa.[2,3] Some of its derivatives were synthesized and their in vitro cytotoxic activities were proved against human esophageal cancer cell line EC9706.[3] Due to their multiple effects, the types of compound have been paid more and more attention recently. They have been reported to exhibit notable cytotoxicity against ovarian cancer cell line,[4] colon cancer cell line,[5] human submandibular gland adenocarcinoma cell line and primary human gingival fibroblast.[6] They could also combat human parasite Trypanosoma cruzi (Chagas's disease)[7] and displayed significant antioxidant activity.[8] To further understand and develop Mahkota Dewa for health and disease treatment applications, in the current study, the known Mahkoside A and a new benzophenone glucopyranoside, 2,4′,6-trihydroxy-4-methoxy-6″-acetyl-benzophenone-2-*O*-β-D-glucoside (Mahkoside B) were isolated from the nut shell of Mahkota Dewa. 16 additional derivatives were also synthesized and their chemical structures were characterized by NMR, HR-MS and UV. The current study is designed to increase the diversity of the compounds and determine the bioactivity of those compounds with different structures. Our goal is to discover a safe and effective new drug with anti-cancer activity and low toxicity from the title plant, through our in vitro screening of the isolated compounds and the related derivatives synthesized. Our results indicate that 14 of the 16 derivatives were synthesized, for the first time, and have been recognized as novel compounds. Their cytotoxicities against four different kinds of cancer cells were also evaluated. Interestingly, we found that compound **18** has significant cytotoxicity against four cancer cell lines with IC_{50} less than 10 μM, indicating it may be a new candidate in our continue searching for anti-cancer drugs.

The mature nut shells of *Phaleria macrocarpa* (Thymelaeaceae) were collected at Pasuruan, East Java, Indonesia in 2007. The botanical identification was made by Professor Zhang Wenhui of the College of Forestry, Northwest Science-Technology, University of Agriculture and Forestry, China. A voucher specimen of the plant was deposited in the 'New Drug Research and Development Center of Zhengzhou University'. The dry nut shell of fruit of *Phaleria macrocarpa* (1900 g) was crushed and extracted by 3 h refluxing for three times with 95% ethanol (2000 ml). The mixture was then evaporated under reduced pressure (50 °C) to obtain the total extract. Then, petroleum ether (1000 ml), chloroform (2000 ml) and ethyl acetate (2000 ml) were added, respectively, for the total products to be further extracted. The acquired ethyl acetate extract was chromatographed on silica gel, using (3:1) or (4:1)

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http://dx.doi.org/10.1016/j.bmcl.2012.09.038

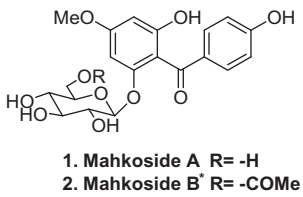


Figure 1. Benzophenone glucopyranosides isolated from the nut shell of Mahkota Dewa. Note: *Indicate that the compound was reported for the first time.

$CHCl_3$/MeOH to give compound **1** (or Mahkoside A, 206 mg), and compound **2** (or Mahkoside B, 17 mg), respectively.

The molecular formula $C_{20}H_{22}O_{10}$ of compound **1** was determined on the basis of EI-MS data together with ^1H and ^{13}C NMR spectra. The UV spectrum suggested that it has a polyphenolic structure. Compound **1** has an identical NMR chemical shift pattern compared with that of the known benzophenone glucopyranoside, Mahkoside A, which had been previously isolated from *Phaleria macrocarpa Gnidia involucrate*.[2] Through detailed analysis of HSQC and HMBC correlations, the identity of compound **1** was identified as Mahkoside A.

The molecular formula $C_{22}H_{24}O_{11}$ of compound **2** was determined on the basis of EI-MS data together with ^1H and ^{13}C NMR spectra. Its UV spectrum is similar to that of compound **1**, suggesting a benzophenone structure again. The ^1H NMR spectrum displayed similar signals besides an additional signal at δ 2.05, integrating for three protons. Two additional corresponding signals at δ 19.91 and δ 170.39 was detected in the ^{13}C NMR spectrum. A HSQC correlation experiment suggested the presence of an acetyl group (^1H: δ 2.05, ^{13}C: δ 19.91 and 170.39). Its position was assigned in C-6″ thanks to an HMBC correlation experiment. Multiplicities were revealed by a DEPT experiment. Gradient HSQC, HMBC and COSY experiments (Fig. 1), allowed complete assignment and identification of the glycone of 2 as 2,4′,6-trihydroxy-4-methoxybenzophenone. The presence of an *O*-β-D-glucose was confirmed by the characteristic ^1H signal for the anomeric proton at δ (4.97, J = 7.7 Hz). Its linkage position at C-2 was ascertained by HMBC. Thus, in the current study, compound **2**, 2,4′,6-tri-hydroxy-4-methoxy-6″-acetylbenzophenone-2-*O*-glucoside, was isolated from the title plant, for the first time, and recognized as a novel compound, which was named as Mahkoside B.

In order to find benzophenone glucopyranoside derivatives with potential anti-cancer bioactivity from the title plan, a variety of structurally modified compounds (**3**–**18**) from three series, including alkylation, acylation and etherification were synthesized, using a nucleophilic substitutional reaction (Fig. 2 and Fig. 3).

For the synthesis of compounds **3**, **4**, **5**, **7**, **8**, **9**, 10 and 11, 206, 102, 136, 122, 126,115,105 and 122 mg Mahkoside A were completely dissolved in 4, 5, 3, 3, 3, 3, 4 and 4 ml DMF, followed by adding 206, 121, 116, 136, 126, 126, 122 and 122 mg anhydrous K_2CO_3. Then, 0.2 ml CH_3I, 0.1 ml CH_3CH_2Br, 0.2 ml $CH_2{=}CHCH_2Br$, 0.1 ml $Br(CH_2)_3CH_3$, 0.2 ml epoxy chlorpropane, 0.2 ml benzyl bromide, 0.2 ml 4-methoxybenzyl chloride and 0.2 ml 3-methoxybenzyl chloride were added dropwise and stirred at 44 °C for 6, 6, 7, 7, 8, 7, 7 and 6 h, respectively. The whole reaction solution was filtered to remove K_2CO_3 and concentrated in vacuum. The following reaction procedures are shown in each compound's synthesis.

For compound **6**, 80 mg Makhoside A was completely dissolved in 3 ml absolute ethyl alcohol, followed by adding 106 mg anhydrous K_2CO_3. Then, 0.1 ml $BrCH_2CH_2CH_2Br$ was added dropwise and stirred at 44 °C for 10 h. The reaction solution was filtered to remove K_2CO_3 and concentrated in vacuum.

For the synthesis of compounds **12**–**15**, 100–140 mg Mahkoside A were completely dissolved in 4 ml pyridine, followed by adding 1.0–2.0 ml acetic anhydride, *n*-butyryl chloride, benzoyl chloride or parachlorobenzoyl chloride dropwise, respectively. The solutions were stirred at room temperature for 0.5 h. Then, 10 ml chloroform was added, stirred and washed with 10 ml distilled H_2O for 3 times. The chloroform solution was concentrated under vacuum to afford compounds **12**–**15**.

For the synthesis of compound **16**, in 25 ml round bottomed flask, 386 mg compound **9** was added to 6.0 ml absolute ethyl alcohol, stirring at 25 °C to fully dissolve the compound. 2.0 ml

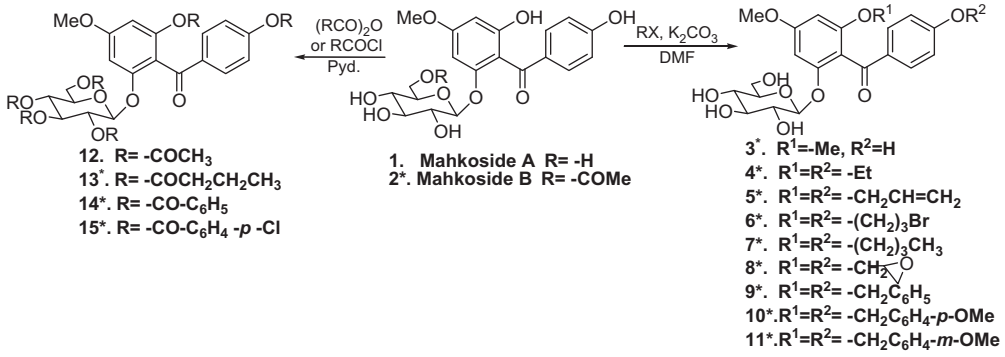


Figure 2. Route of synthesis for compound **3**–**15.** Note: *Indicates that the compounds are reported for the first time.

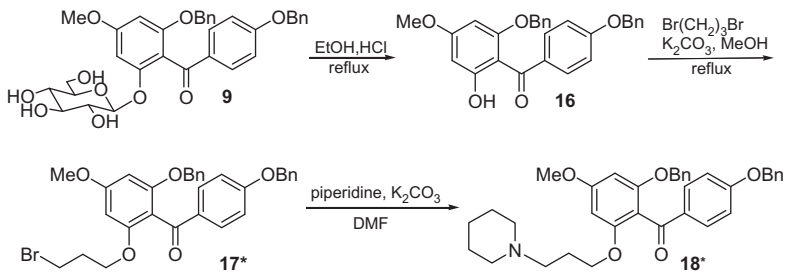


Figure 3. Route of synthesis for compound **16**–**18.** Note: *indicates that the compounds are reported for the first time.

Table 1
Structure of compounds (**1**–**18**) and their effects on cell viability

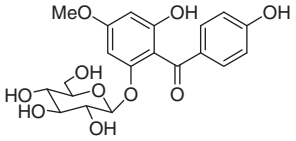
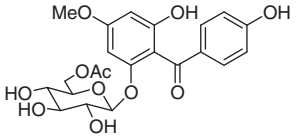
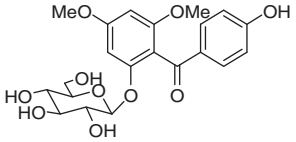
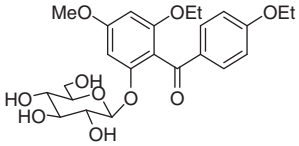
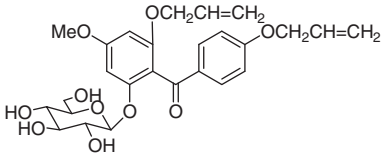
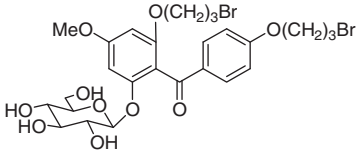
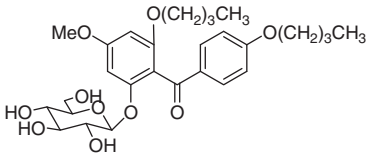
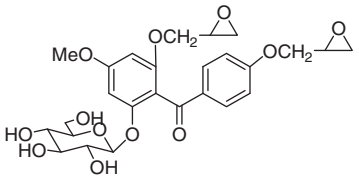
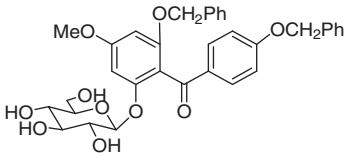
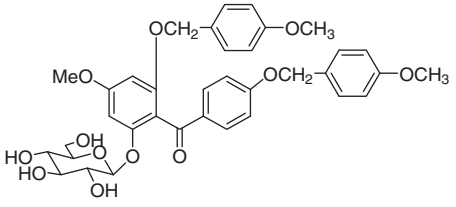
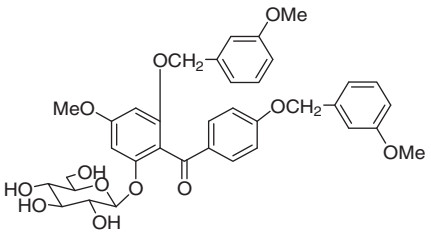
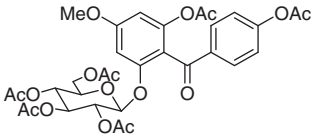
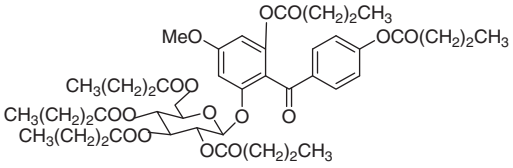
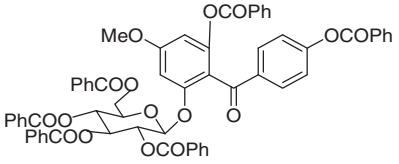
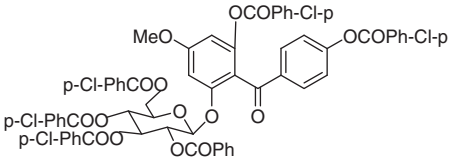
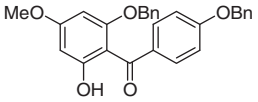
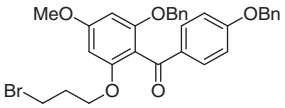
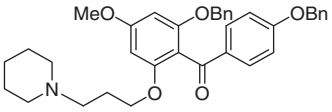
Samples		Yield (%)	Mp (°C)	IC_{50} (μM/L, 72 h)			
				Cell lines			
				EC109	EC9706	MGC-803	PC-3
1			105	>100	>100	>100	>100
2			Oil matter	>100	>100	>100	>100
3		47.9	74	>100	>100	>100	>100
4		96.9	61	>100	>100	>100	>100
5		87.8	67	>100	>100	>100	>100
6		80.6	55	23.057 ± 1.363	31.260 ± 1.495	53.767 ± 1.731	40.000 ± 1.602
7		93.9	52	29.022 ± 1.463	41.763 ± 1.621	49.155 ± 1.692	32.042 ± 1.506
8		80.3	69	>100	>100	>100	>100
9		79.8	62	22.680 ± 1.356	41.062 ± 1.613	44.265 ± 1.646	28.933 ± 1.461
10		75.4	58	17.079 ± 1.232	20.516 ± 1.312	28.180 ± 1.450	11.747 ± 1.070

Table 1 (*continued*)

Samples		Yield (%)	Mp (°C)	IC_{50} (μM/L, 72 h)			
				Cell lines			
				EC109	EC9706	MGC-803	PC-3
11		76.5	59	12.323 ± 1.091	20.852 ± 1.319	18.385 ± 1.264	6.922 ± 0.840
12		91	74	50.662 ± 1.705	86.061 ± 1.935	47.313 ± 1.675	34.063 ± 1.532
13		84	Oil matter	>100	>100	>100	>100
14		72	72	>100	>100	>100	>100
15		77	124	>100	>100	>100	>100
16		78	119	>100	>100	>100	>100
17		96	105	>100	>100	>100	>100
18		58	193	5.139 ± 0.711	4.971 ± 0.696	3.121 ± 0.494	3.474 ± 0.541

Yield (%) and melting points (Mp°C) are indicated for each compound. Inhibition of four different cancer cell growth was expressed as IC_{50} (μM/L). EC109 and EC9706: esophageal cancer cell lines; MGC-803: stomach cancer cell lines; PC-3: prostate cancer cell lines. Three independent experiments were performed in EC109, EC9706, MGC-803 and PC-3 cell lines. Data are Mean ± SEM.

hydrochloric acid (9.5 M) was then added. The temperature was raised to 105 °C and the solution was stirred for another 3 h and ice-bath filtered to obtain the crystals. Absolute ethyl alcohol was used to recrystallize the crystals and afford faint yellow needle crystals of compound **16 (220 mg,** 78% yield).

For the synthesis of compound **17**, in 25 ml round bottomed flask, 206 mg compound **9** was fully dissolved in 10.0 ml absolute ethyl alcohol by stirring at 60 °C, followed by adding 220 mg anhydrous K_2CO_3 and stirred 20 min. 0.5 ml 1,3-dibromopropane was then added dropwise and temperature was raised to 80 °C and stirred for another 2 h. It was further extracted by 10 ml chloroform twice and white needle crystal (252 mg, 96% yield) was obtained.

For the synthesis of compound **18**, in 10 ml round bottomed flask, 96 mg compound **17** was fully dissolved in 4.0 ml DMF by stirring at room temperature. 102 mg anhydrous K_2CO_3 was added and stirred for 20 min. 0.3 ml piperidine was then added dropwise. The solution was stirred 7 h at room temperature and further filtered to remove K_2CO_3. Then, the reduced pressure distillation was performed to remove DMF from the solution. The acquired residue was purified by flash chromatography on silica gel (chloroform/methanol = 20:1) and vacuumized to afford compound **18** (56 mg, 58% yield) as a yellow oil matter.

In order to determine the relationship of structure and activity of the compounds above, several cancer cell lines, including esophageal cancer cell lines: EC109 and EC9706, stomach cancer cell line: MGC-803 and prostate cancer cell line: PC-3, were randomly selected and cytotoxic activity was evaluated according to the standard SRB (sulforhodamine B) assay. Briefly, EC109 and

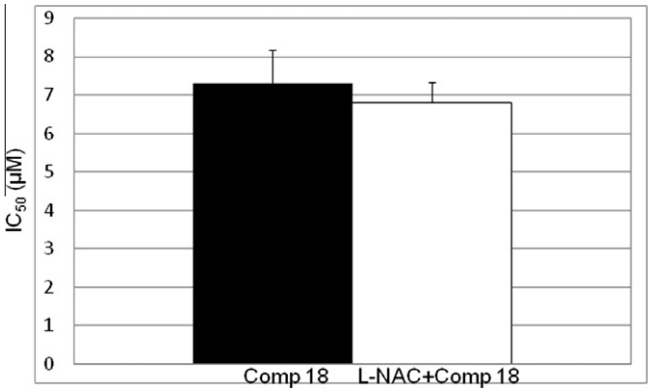


Figure 4. Reactive oxygen species (ROS) inhibitor L-NAC could not block the cytotoxic effect of compound **18**. MGC-803 cells were cultured and treated with or without 5 mM ROS scavenger L-NAC for 2 h before adding compound **18**. SPSS16.0 software was used to analyze the data and calculate the index of 50% cell death (IC_{50}).

EC9706, MGC-803 and PC-3 cell lines, purchased from 'Cell Bank, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences', were cultured in DMEM, containing 10% FBS and 1% penicillin and streptomycin. At the stage of logarithm, cells were washed by PBS and digested with trypsin, then inoculated in 96-well plates by 5000 cells/well. All the compounds used in this study were dissolved in DMSO and made to 10^5 μM/L stock solutions, according to their molecular weights. The final working solutions were acquired by 1000 times dilution of the stock with culture medium, less than 0.1% DMSO was included in each well. The control cells were treated with the same conditions without compounds. After the cells were plated, 200 μl compound solutions with final concentrations of 100, 50, 25, 12.5, 6.25, 3.125 and 1.5625 μM for each compound were added to each well and the cells were incubated for another 72 h. 50% trichloroacetic acid (TCA) were then added to each well to fix the cells for 10 min at room temperature (RT) and 1 h at 4 °C. The plates were then washed five times in distilled water and the cells were allowed to dry in the air. After 0.4% (m/v) SRB (Sulforhodamine B) was added, the cells were incubated at RT for 10 min. Then the SRB solution was changed by quickly washing the plates with 1% v/v acetic acid five times to remove unbound dye. The plates were then dried in the air. To solubilize the bound SRB, 150 μl of 10 mM/L unbuffered Tris Base (pH 10.5) was added to each well and the plates were shaked for 5 min. The optical density (OD) of SRB in each well is directly proportional to the cell number so the OD values can be plotted against concentration, with the working wavelength 515 nm. SPSS16.0 software was used to analyze the data and calculate the index of 50% cell death (IC_{50}).

Interestingly, we found that some of the derivatives showed good inhibition, especially the alkylation derivatives with more than three saturated hydrocarbon lateral chains (compounds **6** and **7**), the phenmethyl derivatives (compounds **9**, 10 and **11**) and acylated derivatives 12. The derivatives with ~OMe replacing groups (compounds **10** and **11**) showed better activity without respect to the position of the replacement. More importantly, we found that compound **18** showed the best cytotoxic activity in four cell lines with IC_{50} less than 10 μM (Table 1). From the structure of compound **18**, it is possible that the substitution of glucoside by azotic alkyl group and the piperidine circle may play an important role in this effect, when compared to compound **17**. To further determine the cytotoxic mechanism of compound **18**, we chose reactive oxygen species (ROS) inhibitor: L-NAC to pre-treat the MGC-803 cells before compound **18** was added, based on our hypothesis of its oxidative cell death effect. To our surprise, L-NAC could not significant impair the effect of compound **18**, indicating that ROS may not play a role in this compound's cytotoxicity (Fig. 4).

In summary, in the current study, two benzophenone glucopyranosides, Mahkosides A and B were isolated from the nut shell of Mahkota Dewa fruit. Using Mahkoside A as a leading compound, 16 derivatives, including alkylation, acylation and etherification substitutions, were synthesized. Among them, 14 were reported, for the first time. The involved cytotoxic activities were also evaluated. Importantly, we found that compound **18** demonstrates significant cytotoxicity against four different kinds of cancer cells. Future studies will be performed to generate and characterize more derivatives with azotic alkyl group and analyze their structure-effect relationships, based on the structure of compound **18**.

Acknowledgment

This work was supported by National Natural and Science Foundation of China (81072539 and 81273393 by Zhang Yan-Bing) and (81270270 by Wen Zhao).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.09.038.

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